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A Method for Studying cAMP-relay in *Dictyostelium discoideum*: the Effect of Temperature on cAMP-relay

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A simple assay has been developed to quantify the cAMP-relay in *Dictyostelium discoideum*. The assay is based on the stimulation of cells, in the presence of a phosphodiesterase inhibitor, with 2'-deoxyadenosine 3',5'-monophosphate (dcAMP) at a concentration which saturates cell surface cAMP receptors. The accumulation of cAMP is measured by an isotope dilution assay using a cAMP-binding protein with such a low affinity for dcAMP that purification of cAMP can be omitted.

The accumulation of extracellular and total cAMP was measured at 20 °C and at 0 °C. At 20 °C the rate of cAMP synthesis increased immediately after stimulation, reaching a maximum after 1 min, with a return to undetectable levels after about 4 min. The secretion of cAMP at this temperature was proportional to the intracellular cAMP concentration and reached a maximum at about 2 min. At 20 °C the cAMP-relay was essentially completed within 3–4 min, yielding about 35 pmol cAMP per 10^7 cells. At 0 °C the same amount of cAMP was relayed, but the rate of cAMP synthesis was reduced about 2·5-fold. The secretion rate depended on the intracellular cAMP concentration with a delay period of 1·25 $\pm$ 0·5 min, and was reduced about 5·5-fold compared to secretion at 20 °C. Due to the relatively slow secretion and fast synthesis of cAMP, an approximately twofold higher intracellular cAMP concentration was reached at 0 °C compared to 20 °C, and this persisted for about 15 min. Taking into account that the secretion rate is proportional to intracellular cAMP concentration, these results indicate that the mechanism underlying cAMP secretion is slowed 13-fold when the temperature is reduced from 20 °C to 0 °C.

INTRODUCTION

Cell aggregation in the cellular slime mould *Dictyostelium discoideum* is mediated by chemotaxis to cAMP (Konijn *et al.*, 1967), which is secreted in pulses with a periodicity of 5 to 10 min (Shaffer, 1975; Gerisch & Wick, 1975). Cells detect cAMP by means of cell surface receptors (Malchow & Gerisch, 1974), which induce three responses: (i) a movement step in the direction of the highest cAMP concentration (chemotaxis); (ii) the synthesis and secretion of cAMP (relay); (iii) termination of chemotaxis and relay by adaptation (Dinauer *et al.*, 1980*a*, *b*, *c*; Van Haastert & Van der Heijden, 1983; Van Haastert, 1983*a*). The dynamics of cAMP sensing, chemotaxis, cAMP-relay and adaptation underlie the pulsatile nature of cell aggregation in *D. discoideum* (Tomchik & Devreotes, 1981).

The analysis of cAMP-relay is complex because the response (cAMP-secretion) interferes with the cAMP stimulus. Devreotes and co-workers (Devreotes *et al.*, 1979; Devreotes & Steck, 1979; Dinauer *et al.*, 1980*a*, *b*, *c*) have circumvented this feedback loop by stimulating the cells while the secretion products are continuously removed in a perfusion apparatus. Their experiments revealed that cells only synthesize cAMP as long as the cAMP stimulus is present. Furthermore, cells respond to an increase of the cAMP stimulus and adapt within a few minutes to constant stimuli. The rate of cAMP secretion is proportional to the intracellular cAMP concentration.

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The perfusion method for the analysis of cAMP-relay was essential to elucidate the mechanism of the relay response. However, it is inconvenient for routine analysis and screening programmes because of the requirement for purification of the secretion product and because of the limited number of positions in the perfusion apparatus. In this report a simple method for measuring cAMP-relay is presented in which cells are stimulated in suspension by a saturating concentration of a potent cAMP-agonist which does not interfere with subsequent detection of cAMP in unpurified extracts by isotope dilution assay.

It has been shown previously that oscillations of *D. discoideum* cells in suspension (Wurster, 1976) or on an agar surface (Nanjundiah *et al.*, 1976) are temperature-dependent with an activation energy (ΔE) of 16 kcal mol^{-1} (65 kJ mol^{-1}). ΔE can be interpreted as the activation energy of a rate-limiting step in the overall scheme of the biochemical oscillation, such as cAMP sensing, intracellular cAMP synthesis or cAMP secretion, or as another step in the network to the measured response (shape change or amoeboid movement). Assuming that the linear Arrhenius plot observed between 20 °C and 8 °C remains linear down to 0 °C, ΔE = 65 kJ mol^{-1} indicates a sevenfold reduction in the frequency of oscillations between 20 °C and 0 °C. The dcAMP assay for cAMP-relay has been used to measure the kinetics of cAMP synthesis and secretion at 20 °C and at 0 °C. The results suggest that cAMP secretion rather than cAMP sensing or cAMP synthesis is the temperature-dependent, rate-limiting step during cAMP oscillations in *D. discoideum*.

METHODS

2′-Deoxyadenosine 3′,5′-monophosphate (dcAMP), and adenosine 3′,5′-monophosphate (cAMP) were obtained from Boehringer Mannheim; [2,8-^{3}H]cAMP (0·9 TBq mol^{-1}), and the cAMP-binding protein kit were purchased from Amersham.

Dictyostelium discoideum NC-4(H) was grown and harvested as described previously (Van Haastert & Van der Heijden, 1983). Cells were starved in buffer or on non-nutrient agar; both methods yield essentially identical results.

Binding of cAMP to cell surface receptors was measured by the ammonium sulphate stabilization assay (Van Haastert & Kien, 1983).

cAMP-relay was measured as follows. Cells were starved for 4·5 h, washed twice in 10 mM-KH_2PO_4/Na_2HPO_4, pH 6·5, and resuspended at a density of 6·25 × 10^7 cells ml^{-1}. During the entire experiment air was bubbled through the cell suspension at a rate of about 15 ml air (ml suspension)$^{-1}$ min^{-1}. Experiments at 0 °C were done in melting ice in a cold room (2 °C); cells were aerated at 0 °C for 10 min before stimulation. One volume of the cell suspension was stimulated with one-quarter volume dcAMP yielding final concentrations of 5 × 10^7 cells ml^{-1}, 10 μM-dcAMP, 5 mM-dithiothreitol (an inhibitor of phosphodiesterase) in 10 mM-phosphate buffer. Total cAMP was measured by transfer of 100 μl cell suspension to tubes containing 100 μl perchloric acid (3·5%, v/v). Extracellular cAMP was measured by brief (5 s) centrifugation at 8000 *g* of 110 μl cell suspension, and transfer of 100 μl of the supernatant to perchloric acid. The lysates were neutralized by 50 μl $KHCO_3$ (50% saturated at 20 °C), and cAMP was measured by isotope dilution assay using a commercial assay kit, or purified regulatory subunit of cAMP-dependent protein kinase type I.

The accuracy of the method (four determinations in one experiment) was 6% for plateau values, 10% for steep ascending values, and 16% for basal values. The reproducibility of 14 independent experiments was 38 ± 12 pmol cAMP per 10^7 cells for the plateau values. When data are normalized as percentage of the plateau value, then the method is highly reproducible, e.g. 50% accumulation of total cAMP at 20 °C was at 1·25 ± 0·1 min (mean ± SD; n = 14).

RESULTS

dcAMP is a potent agonist of cAMP in *D. discoideum* (Konijn, 1974; Mato *et al.*, 1978; Van Haastert, 1983*b*). The compound is five- to tenfold less active than cAMP at inducing a chemotactic response or competing with [^{3}H]cAMP for binding to cell surface receptors (Fig. 1). It has been shown previously that dcAMP has a very low affinity for the regulatory subunit of protein kinase (De Wit *et al.*, 1982). In the commercial isotope dilution assay dcAMP was about 1500-fold less active than cAMP (Fig. 1), and this was also true for binding to protein kinase. Thus, stimulation of *D. discoideum* cells with 10 μM-dcAMP, equivalent to about 1·4 μM-cAMP, saturates the cell surface receptors and induces an almost maximal cAMP-relay response. The

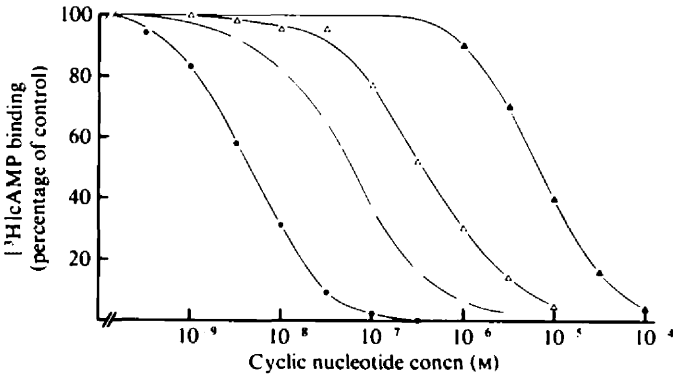

Fig. 1. Specificity of cell surface cAMP receptors of *D. discoideum* (○, △) and bovine heart cAMP-protein (●, ▲) for cAMP (○, ●) and dcAMP (▲, △). Aggregative *D. discoideum* cells were incubated with 10^{-8} M-[^{3}H]cAMP and different concentrations of cAMP or dcAMP, and residual [^{3}H]cAMP binding was measured with the ammonium sulphate stabilization assay (Van Haastert & Kien, 1983). The specificity of the commercial cAMP-binding protein was tested as described by the manufacturer. Essentially identical results were obtained if the beef heart cAMP-binding protein (which is not further specified) was replaced by the regulatory subunit of cAMP-dependent protein kinase.

amount of cAMP secreted was typically 0·15 μM, which is well below the stimulus strength; therefore, the stimulus–response feedback loop had been by-passed by using high concentrations of dcAMP. In the isotope dilution assay 10 μM-dcAMP is equivalent to only 7 nM-cAMP, or about 1·4 pmol per 10^7 cells.

The time course of cAMP accumulation and cAMP secretion at 20 °C and at 0 °C in aggregative *D. discoideum* cells is shown in Fig. 2. At 20 °C cAMP increased after stimulation with dcAMP, reaching a maximum after 3–4 min, followed by a small but reproducible decrease of total cAMP between 4 and 6 min. This decrease may represent intracellular degradation of cAMP (Dinauer *et al.*, 1980*a*). The delay between intra- and extracellular cAMP was about 30–45 s. The accumulation and secretion rates were calculated by taking the first derivative of, respectively, the total and the extracellular cAMP concentration. The cAMP accumulation rate increased immediately after the addition of the stimulus; the maximal level was reached after about 1 min. The cAMP secretion rate increased more slowly, and the maximal value was obtained later (2 min). The cAMP-relay response at 20 °C was essentially complete within about 5 min.

At 0 °C approximately the same amount of cAMP was relayed as at 20 °C. However, accumulation of cAMP at 0 °C was slower than at 20 °C; the maximal cAMP accumulation rate was about 2·5-fold less and was obtained about 2·5 times later. cAMP secretion at low temperatures was reduced more than cAMP accumulation; the maximal secretion rate at 0 °C was about 5·5-fold less than at 20 °C.

Dinauer *et al.* (1980*a*) have shown that the cAMP secretion rate is proportional to the intracellular cAMP concentration with a coupling factor (K_s) of 0·34 min^{-1} or 0·94 min^{-1} for the two experiments shown. In the present report intracellular cAMP concentrations were calculated by substracting extracellular levels from total levels. There was a good correlation between intracellular cAMP and the secretion rate (Fig. 2*c*: $r = 0{\cdot}96$, $n = 8$, $P < 0{\cdot}001$). The K_s at 20 °C is 1·44 min^{-1}. Linear regression analysis of the correlation between intracellular cAMP and the secretion rate at 0°C yields $r = 0{\cdot}88$, $n = 9$, $P < 0{\cdot}01$; Fig. 2*d*). The K_s calculated by linear regression analysis is 0·11 min^{-1}, which is 13-fold less than at 20°C.

The data presented in Fig. 2(*d*) show that at 0 °C the observed secretion rate at increasing intracellular cAMP concentrations was less than the secretion rate expected from linear regression analysis. The contrary is observed at decreasing cAMP concentrations; the observed secretion rate is greater than expected. This indicates a time delay between the response of

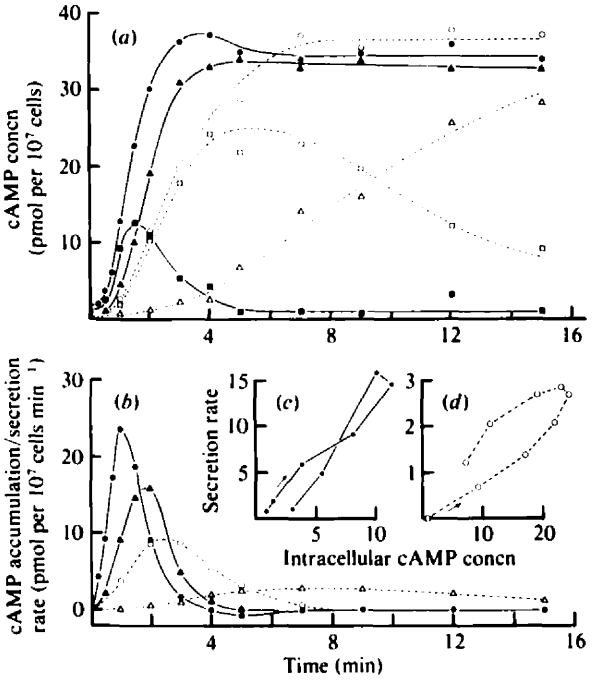

Fig. 2. cAMP-relay in *D. discoideum* at 20 °C and 0 °C. (*a*) A suspension of 3 ml aggregative *D. discoideum* cells was stimulated with 0·75 ml dcAMP. Total cAMP was determined by transfer of 100 µl suspensions at the indicated times to tubes containing 100 µl perchloric acid. Extracellular cAMP was determined by centrifugation of 110 µl suspensions for 5 s at 8000 *g* and transfer of 100 µl supernatant to 100 µl perchloric acid; centrifugation was started at 5 s before the indicated times and lasted 15–20 s. The cAMP concentration was measured in the neutralized lysates. Filled symbols, 20 °C; open symbols, 0 °C. ●, ○, Total cAMP; ▲, △, extracellular cAMP; ■, □, intracellular cAMP calculated from total and extracellular levels. (*b*) The rates of cAMP accumulation and cAMP secretion were calculated by taking the first derivatives of the curves representing, respectively, total cAMP and extracellular cAMP. Filled symbols, 20 °C; open symbols, 0 °C. ●, ○, Accumulation rate; ▲, △, secretion rate. (*c*, *d*) Rate of cAMP secretion (pmol cAMP per 10^7 cells min^{-1}) plotted against the intracellular cAMP concentration (pmol per 10^7 cells) (from which a prestimulation level of 1 pmol per 10^7 cells was subtracted). The sequence of data during the relay response is connected; the arrow indicates the onset of the response. (*c*) 20 °C; (*d*) 0 °C. The results are the means of three independent experiments normalized to their plateau values at 5–10 min at 20 °C (respectively, 44, 30 and 28 pmol cAMP per 10^7 cells).

intracellular cAMP concentrations and that of the secretion rate. A comparison between the secretion rate expected for a certain intracellular concentration and the time at which this secretion rate is observed indicates that the delay at 0 °C is 1·25 ± 0·5 min. The data at 20 °C yield an insignificant delay time of 0·05 ± 0·17 min.

DISCUSSION

cAMP induces the synthesis and secretion of cAMP in *D. discoideum* cells. Investigation of the mechanism of the relay response requires a method in which the stimulus-secretion feedback loop is eliminated. In the perfusion method developed by Devreotes *et al.* (1979) [^{3}H]adenosine-labelled cells on Millipore filters are perfused with buffer. Radiolabelled products in the perfusate are then extensively purified to yield the amount of secreted cAMP.

In this report a simple method for measuring cAMP-relay is described which does not require labelling of the cells with a precursor of cAMP or the purification of secretion products. Cells are stimulated in suspension with a concentration of dcAMP sufficient to saturate the cAMP

receptor, in the presence of a phosphodiesterase inhibitor. cAMP is subsequently measured by isotope dilution assay using a binding protein which has very low affinity for dcAMP. This method is especially useful if cells are stimulated under many different conditions (e.g. pH, pharmacological agents), since the procedure does not require a perfusion apparatus with a limited number of positions. The dcAMP method is not suitable for investigating adaptation of cAMP-relay, because of the dcAMP stimulus cannot be easily manipulated. The time course and magnitude of cAMP accumulation and secretion induced by dcAMP in suspended cells at 20 °C are qualitatively and quantitatively similar to those induced by cAMP in cells on filters (Dinauer *et al.*, 1980*a*, *b*, *c*).

The experiments on cAMP-relay at 0 °C reveal that cells are fully able to respond at this low temperature. Approximately equal amounts of cAMP are secreted at 0 °C and at 20 °C. The cAMP accumulation rate (which is probably very similar to the rate of cAMP synthesis) is reduced at 0 °C only 2·5-fold. The rate of cAMP secretion is reduced about 5·5-fold at 0 °C. This suggests that the approximately sevenfold decrease in the rate of cAMP oscillations in suspension (Wurster, 1976) or on an agar surface (Nanjundiah *et al.*, 1976) is more probably due to delayed cAMP secretion than to slower cAMP sensing or cAMP synthesis. At both 0 °C and 20 °C the rate of cAMP secretion is closely correlated with the intracellular cAMP concentration, although secretion at 0 °C follows intracellular cAMP with a time delay of 1·25 ± 0·5 min. It is likely that a time delay may also exist at 20 °C; however, previous (Dinauer *et al.*, 1980*a*) and present data indicate that the delay is too short to be detectable (less than 15 s in the present report).

The approximately twofold higher intracellular cAMP concentrations at 0 °C combined with the 5·5-fold slower secretion rate indicate that the mechanism underlying cAMP secretion is slowed 13-fold by lowering the temperature from 20 °C to 0 °C (this value is obtained from the ratio of K_s coupling factors). Apparently, cAMP secretion proceeds via a mechanism which is strongly temperature-dependent. The reduced fluidity of membranes at 0 °C could be the cause of slow cAMP secretion. The hypothesis that cAMP is secreted by fusion of cAMP-containing vesicles with the plasma membrane (Maeda & Gerisch, 1977) would also explain the long-term storage of cAMP at 0 °C.

The observation that cAMP-mediated cAMP synthesis is hardly affected by reduced temperatures, and that cAMP secretion is largely impaired at 0 °C may help to elucidate the secretion mechanism and intracellular localization of cAMP during the relay response.

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